SUPPLEMENTARY DATA

Liquid Chromatography-mass spectrometry (LC-MS). To identify the phosphorylation sites of Foxo1, we performed LC-MS/MS analysis. The specific bands on the SDS-PAGE gel after Coomassie-brilliant blue staining were excised, cut into several pieces, and subjected to dimethyl formamide (DMF)-assisted trypsin digestion. The digested peptides were analyzed by LC-MS/MS. Briefly, the pieces of the gel was dissolved in 50 mM NH_4HCO_3 buffer (pH8.0) containing 40% DMF and added with 20 μl of 2 μg trypsin, and incubated overnight at 37 °C. After digestion, the solution was further diluted by adding three-fold volume of deionized water. After centrifugation, the supernatant was collected. The debris were washed in 80 μL of 70% acetonitrile and 1% rifluoroacetic acid with sonication for 30 min and then centrifuged. Both supernatants were combined and concentrated in a Speed-Vac and stored at -20 °C until further use. To identify the unknown proteins and phosphorylation sites (p-sites), HPLC conjugated electrospray ionization (ESI) ion trap mass spectrometry were employed. The samples were separated on an Alltech Vydac MS C18 column (300 A, 5μm, 100 mm × 300 μm) at a flow rate of 5μl/min using a mobile phase of (A) 0.1% v/v formic acid in water and (B) 0.1% v/v formic acid in acetonitrile. The chromatography system was directly coupled to an ESI Ion-trap mass spectrometer. Raw spectrum data were processed and MASCOT-compatible mgf files were created using DataAnalysis software (Bruker Daltonics). Peptide searching and analysis were performed using MASCOT software (Matrixscience, London, UK). The NCBI gene bank database was used for protein identification.

Generation of phosphospecific antibodies for Foxo1. The conjugated peptides were used to immunize rabbits. Six weeks later serum was collected and passed through a CH-sepharose column containing beads coupled to dephospho-peptide antigen, followed by affinity chromatography on the column with beads covalently coupled to phosphor-peptide antigen. Phosphospecific antibodies were eluted with 0.1 M glycine, pH 2.4, immediately adjusted to pH 8.0 with Tris-base and stored at -20ºC. The phosphospecific antibodies was passed quality control tests, in which 50% titer Elisa test was used to determine signal intensity. The reference of 153,000 for antibody against pFoxo1-S276 and 105,000 for antibody against pFoxo1-S153 achieved, indicative for high specificity signals to corresponding specific phosphorylation sites, in comparison with control non-phosphorylated peptides as reference of signal less than 100 .

Chemicals. Insulin, 8-Br-cAMP, glucagon, [des-His^1, Glu^9]-Glucagon amide, MG132, and CHX were purchased from Sigma; Foxo1, pFoxo1-S256, Akt, pAkt-T308, PKA, pPKA-T197, and β-actin antibodies were from Cell Signaling Technology. Antibodies specific for CREB, pCREB-S133, Histone H1, and β-actin were from Santa Cruz. The scramble siRNA (AM4611) and siRNA-PKACB (4390824) were purchased from Thermo Fisher (Waltham, MA).

PKA knock-down assay. HepG2 cells were cultured with DMEM with 10% FBS for 6 h. Cell culture were then subjected to Lipofectamine® 3000 (Life technologies) with scramble siRNA or siRNA-PKACB according to manufacturer's instruction. 24 h after transfection, cells were subjected to further treatment.

Generation of Foxo1-S273A and Foxo1-S273D Knock-in mice. Mouse Foxo1 gene (Ensembl: ENSMUSG00000044167) is located on mouse chromosome 3. Three exons have been identified, with the ATG start codon in exon 1 and TAA stop codon in exon 2. The Foxo1-S273 is located in exon 2 and selected as target sites. The gRNA was designed and the targeting vectors constructed, and oligo donors with targeting sequence flanked by 60bp homologous sequences were designed. The S273A (TCT to GCT), S273D (TCT to GAT) mutation site in the donor oligo was introduced into exon 2 by homology-directed repair. The Cas9 mRNA and gRNA were generated by *in vitro* transcription and oligo donor was co-injected into fertilized eggs for the KI mouse production.

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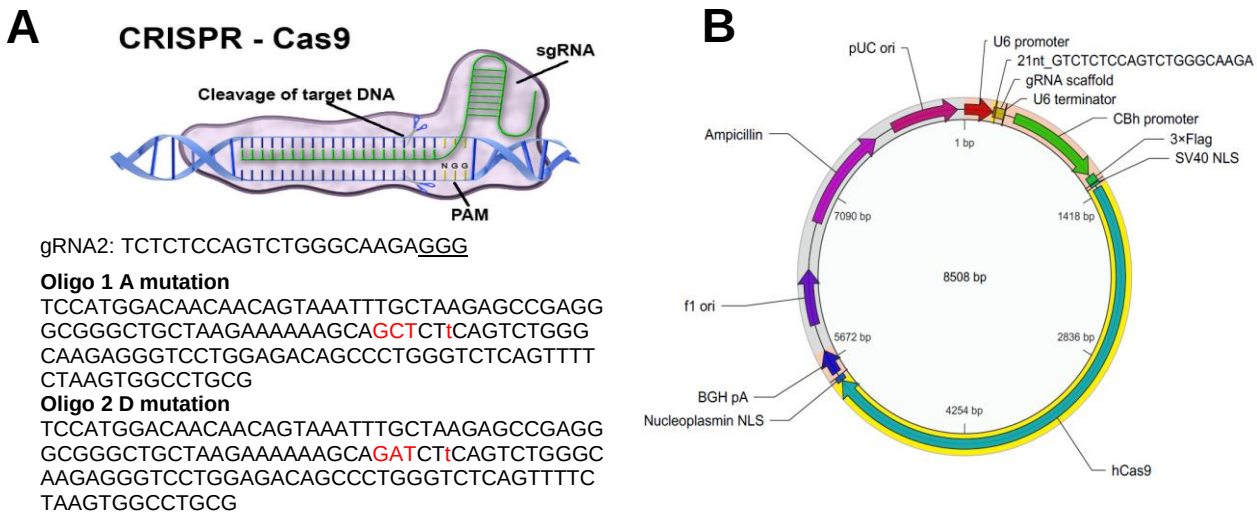
SUPPLEMENTARY DATA

Supplementary Figure S1. DNA sequencing of Foxo1-S273A and Foxo1-S273D heterozygotes and homozygotes in mice. (**A**) Construction of plasmid DNA expressing Foxo1 guidance RNA with human Cas9 expression vector. (**B**) Sequencing results of Foxo1 alleles from +/+ (wild-type or WT), A/+, and D/+ heterozygous founder mice. (**C**) The breeding strategy and genotyping for Foxo1-S273$^{+/+}$, S273$^{A/A}$, and S273$^{D/D}$ alleles in mice.



gRNA2: TCTCTCCAGTCTGGGCAAGAGGG

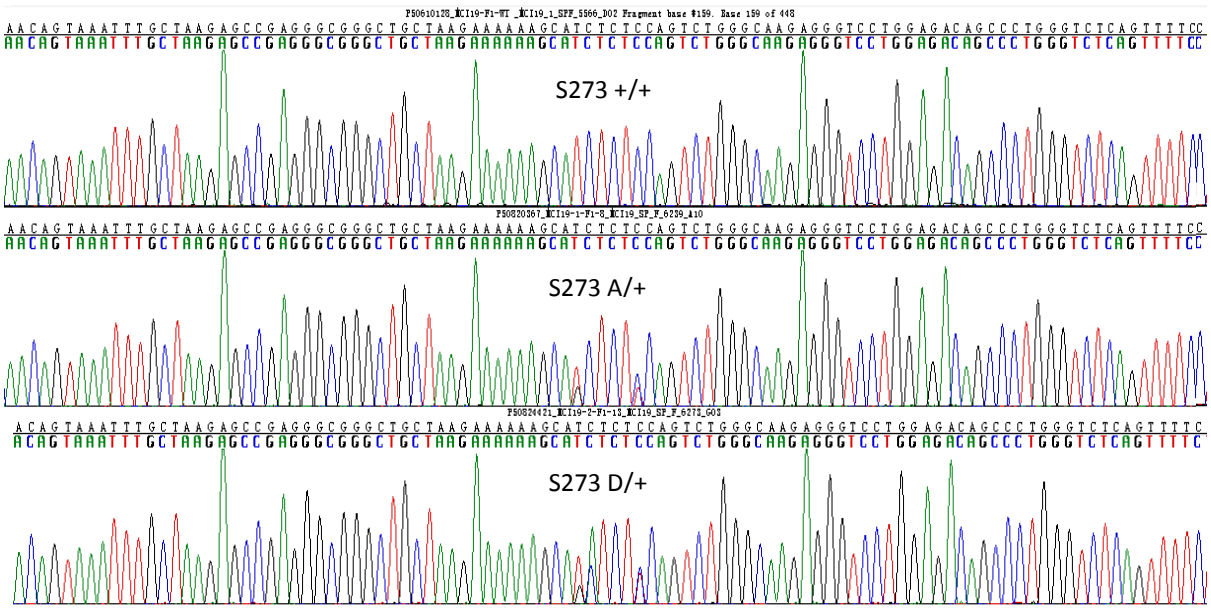
Oligo 1 A mutation
TCCATGGACAACAACAGTAAATTTGCTAAGAGCCGAGG
GCGGGCTGCTAAGAAAAAAGCAGCTCTtCAGTCTGGG
CAAGAGGGTCCTGGAGACAGCCCTGGGTCTCAGTTTT
CTAAGTGGCCTGCG
Oligo 2 D mutation
TCCATGGACAACAACAGTAAATTTGCTAAGAGCCGAGG
GCGGGCTGCTAAGAAAAAAGCAGATCTtCAGTCTGGGC
AAGAGGGTCCTGGAGACAGCCCTGGGTCTCAGTTTTC
TAAGTGGCCTGCG

C

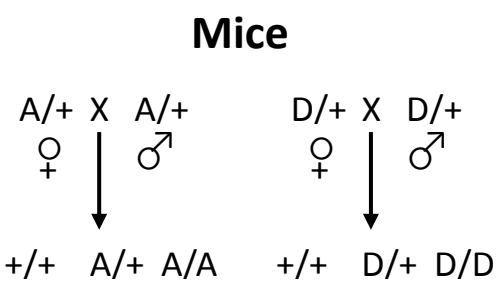
DNA Sequencing Primer (Forward Sequencing): 5'-

Specific primers: mFoxo1-gRNA-SPF:

GGGGGAGTGCTGCACTTCTAACATA



D



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